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Homocysteine as a Risk Factor for the Development of Cardiovascular Disease; the Role of NOX Proteins

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GENERAL DISCUSSION

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Since 1962¹ it has become apparent that increased levels of homocysteine (Hcy), also known as hyperhomocystenaemia (HHC), form a risk factor in the development of neurological disorders such as Parkinson disease² and Alzheimer^{3, 4}, but also in cancer^{5, 6}, preeclampsia⁷ and cardiovascular disease⁸⁻¹⁶. Recent studies indicate that NADPH oxidase (NOX) forms a new source of reactive oxygen species (ROS) within the cardiovascular system and as such plays an important role in the development of cardiovascular disease in general. In this thesis we have studied the role of NOX in Hcy-induced cardiovascular disease with specific emphasis on two important cell types, cardiomyocytes and endothelial cells. Although the role of Hcy as a primary inducer of cardiovascular disease has been debated in recent years, there is ample evidence that high Hcy levels occur in (even young) patients with cardiovascular disease. This suggests that Hcy accelerates or aggravates the disease in patients who are developing atherosclerosis. Furthermore, limited information is available regarding the effect of Hcy on cardiomyocytes.

Homocysteine pathway (Figure 1)

Methionine normally is converted to S-adenosylmethionine (SAM; **1**). Donation of the methylgroup to methyltransferase then gives rise to SAH (**2**). This in turn is hydrolysed to adenosine and Hcy (**3**) by SAH hydrolase (SAHH). Remethylation of Hcy to methionine (**4**) occurs, using co-factors folate and vitamin B12, when there is a demand for SAM¹⁷. If SAM levels are sufficient, transsulfuration occurs (**5**), using vitamin B6 as a co-factor to form cysteine and glutathione^{17, 18}.

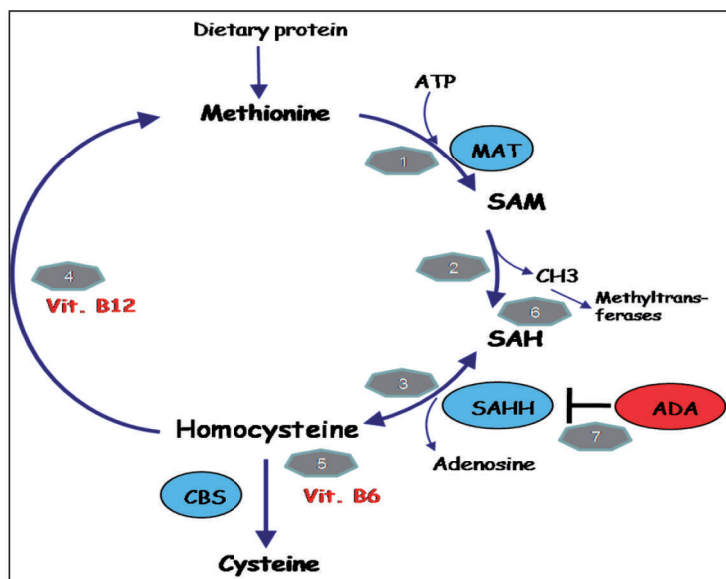


Figure 1. Homocysteine pathway. Homocysteine as part of the methionine cycle.

NADPH oxidase (NOX) complex (Figure 2)

The NADPH oxidase (NOX) complex has been studied extensively in neutrophilic granulocytes¹⁹⁻²³. From these cells it is known that the components of the NADPH oxidase are distributed between the cytosol (p67^{phox}, p47^{phox}, p40^{phox}, Rac2) and the membrane (gp91^{phox}/NOX2 and p22^{phox}). Upon cell activation, the cytosolic components p47^{phox}, p67^{phox}, and p40^{phox} (1) are phosphorylated and migrate to the membranes where they associate with gp91^{phox} and p22^{phox} (2). At the same time, Rac2 exchanges its GDP to GTP (3), dissociates from its inhibitor Rho-GDI, and migrates to the membrane. Cytochrome b558, which contains both flavin and heme groups, is then activated by p67^{phox} via its activation domain and Rac2. Activated NADPH oxidase then uses cytosolic NADPH (4) to reduce oxygen and to produce superoxide anion²⁴(5). Meanwhile different cell-specific NOX isoforms have been described, also in cardiovascular cells. As such NOX1, NOX2, NOX4, NOX5 and DUOX1/DUOX2 have been identified in cardiomyocytes and/or vascular cells²⁵⁻²⁷, each with different cytosolic components. The effect of Hcy on cardiovascular NOX however is less known.

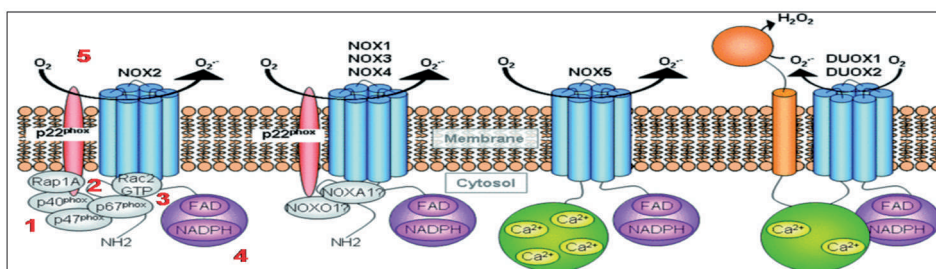


Figure 2. NADPH (NOX) complex. The different NOX subunits and isoforms are depicted with their interactions. N.B. the phox subunits of the complex also react with NOX1, NOX3 and NOX4 but were left out to clarify differences between the isoforms.

Hcy and Endothelial Cell Dysfunction (Figure 3)

Endothelial dysfunction, including induction of cell death, plays a major role in the process of vascular injury during HHC^{28-31, 31-35}. In animal models it was shown that Hcy induced apoptosis of endothelial cells, in which ROS plays an important role³⁶⁻⁴⁰. Additional studies have shown that apoptosis induction in endothelial cells by Hcy in part is regulated via NOX, without further specifying the NOX isoform herein^{31, 41-43}. Since we have shown that translocation of NOX2 to the nucleus is crucial for apoptosis induction in ischemic cardiomyocytes⁴⁴ we have analyzed whether this nuclear NOX-mediated apoptosis pathway also plays a role in apoptosis induction of endothelial cells by Hcy.

Incubation of endothelial cells with 2.5 mM D,L-Hcy during 6 hours not only induced cell death, visualized by propidium iodide (PI), entering endothelial

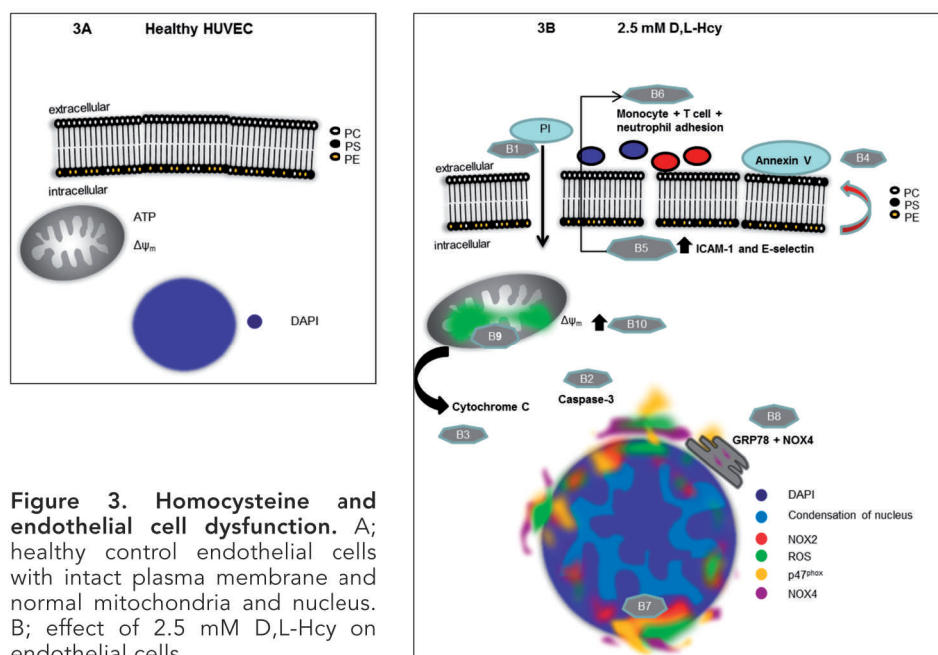


Figure 3. Homocysteine and endothelial cell dysfunction. A; healthy control endothelial cells with intact plasma membrane and normal mitochondria and nucleus. B; effect of 2.5 mM D,L-Hcy on endothelial cells.

cells (B1), but also resulted in activation of caspase-3 (B2), indicative of apoptosis. Coinciding with increased caspase-3 activation, we also found an increase in cytochrome C release from the mitochondria, underlining apoptosis induction (B3). Interestingly we also observed that Hcy induced externalization of phosphatidyl-serine (PS) from the inner to the outer layer of the plasma membrane, the so-called plasma membrane flip-flop, as measured via binding of fluorescent labelled Annexin V to these membranes (B4). This flip-flop can occur in apoptotic cells, but can also be found in cells that are non-apoptotic, representing a reversible, but pro-inflammatory status of the cell⁴⁵⁻⁴⁷. It indeed is known that Hcy activates endothelial cell adhesiveness through e.g. an increase in intercellular adhesion molecule-1 (ICAM-1) expression and E-selectin induction (B5)⁴⁸⁻⁵⁴, as such facilitating binding of monocytes, T-lymphocytes but also neutrophilic granulocytes to endothelial cells (B6)⁵⁴⁻⁵⁷.

Since NOX2 and NOX4 are the most abundant NOXs in endothelial cells^{27, 58}, we examined their expression pattern along with the NOX2 activation component p47^{phox}. We found that 2.5 mM D,L-Hcy induced (peri)nuclear co-localization of NOX2, NOX4 and p47^{phox} coinciding with (peri)nuclear ROS production. (B7). The question then arises what the function of this (peri)nuclear NOX/ROS is. Chen et al.⁵⁹ have found peri-nuclear NOX4 localization in control endothelial cells, co-localizing with the ER marker protein GRP78, indicative for a role of NOX in redox signalling at the ER (B8). Interestingly, it has also been shown that ROS can activate the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinases (MAPKs) and Akt, in endothelial

cells treated with H_2O_2 ⁶⁰. Whether NOX proteins, regarding their particular (peri-) nuclear localization, can activate or modulate transcription factors^{61, 62} is however still unknown. Notwithstanding this, we did find a role of NOX in the process of apoptosis induction in endothelial cells. Co-incubation of Hcy with the NOX inhibitor diphenylene iodonium (DPI) namely resulted in a decrease in cytochrome C release and caspase-3 activity indicating that induction of apoptosis by Hcy is NOX related. We however also found that Hcy induced increased cytoplasmic levels of ROS, not co-localizing with NOX2 or -4, that theoretically could be derived from mitochondria³⁵. Inhibition of mitochondrial ROS using rotenone then indeed did also inhibit apoptosis.

Although an early marker of apoptosis of the mitochondrial pathway is a decrease in the mitochondrial membrane potential $\Delta\Psi_m$, we surprisingly found that 2.5 mM D,L-Hcy induced a significant increase in this mitochondrial membrane potential. This would implicate actively respiring mitochondria, coinciding with increased ATP levels, whereas the reverse is normally associated with apoptosis (**B10**). This therefore could represent a secondary cell protective mechanism related to Hcy, although this was still inadequate regarding the induction of apoptosis at that particular concentration. We are aware of the fact that the *in vitro* concentrations of Hcy (up to 2.5 mM) we have used are relatively high compared to its (patho-) physiological concentrations (up to 400 μ M). However, we have also shown that Hcy is rapidly degraded *in vitro*. As such the short-term exposure (6 and 24 hours) to higher Hcy concentrations we used might reflect a life-long exposure to moderately elevated levels of Hcy, as occurs in patients. Furthermore, patients suffering from homocystinuria revealed plasma Hcy levels ranging from 200-400 μ M⁶³. As we have found that 2.5 mM D,L-Hcy represents only 1.2 mM of L-Hcy (only the L-form of Hcy has a biological effect, not the D-form^{50, 64}), this indicates that we in fact studied up to a 3-fold higher concentration than physiologically occurs in these patients^{50, 64}.

There is also an ongoing debate whether Hcy or increased S-adenosylhomocysteine (SAH), a very potent inhibitor of methylation (**Figure 1**) (**6**) of DNA, RNA and proteins, is the main causative factor in HHC induced cardiovascular disease⁶⁵⁻⁶⁷. Therefore, we examined the effect of S-adenosylhomocysteine (SAH) on endothelial cell viability also. For this we inhibited SAH hydrolase (SAHH) with adenosine-2,3-dialdehyde (ADA; **Figure 1**)(**7**), resulting in accumulation of SAH in the cells, while the formation of Hcy was inhibited. We then found that increased intracellular SAH induced both plasma membrane flip-flop and apoptosis, coinciding with (peri)nuclear NOX2/NOX4/p47^{phox} and with local ROS production, that was comparable with Hcy incubation of 2.5 mM during 6 hrs. From these findings we can conclude that increased levels of intracellular SAH, also can induce endothelial cell damage, next to Hcy.

In conclusion, we have shown that both Hcy and intracellular SAH not only induced endothelial cell apoptosis via (peri)nuclear NOX2/NOX4/p47^{phox} induced ROS production but also a pro-inflammatory status, visualized via the so-called plasma membrane flip-flop induction.

Homocysteine and the Heart (Figure 4)

Until now, Hcy related cardiovascular disease mainly was studied in relation to its effect on vascular cells. However, recent studies also suggest a patho-physiological effect of HHC on cardiomyocytes, e.g. resulting in cardiac fibrosis and secondary hypertrophy of cardiomyocytes⁶⁸⁻⁷¹. These fibrotic changes in part can be explained by an effect of Hcy on the extracellular matrix (ECM) via activation of matrix metalloproteinases (MMP's) and inhibition of the tissue inhibitors of metalloproteinases (TIMP's)⁷²⁻⁷⁷. However, unknown is whether Hcy is also cytotoxic for cardiomyocytes, inducing cell death that could also explain (secondary) cardiac fibrosis. .

We now found that incubation of the cardiomyoblast cell-line (H9c2 cells) with 0.1 mM D,L-Hcy during 24 hours, resulted in an increase in ATP and mitochondrial membrane potential (**A1**). Theoretically this could represent a cell-protective mechanism⁷⁸. However, at the same time we found translocation of NOX2 to the nuclear area (**A2**), albeit without ROS generation. Apoptosis or flip-flop induction of the plasma membrane was neither found. When the concentration

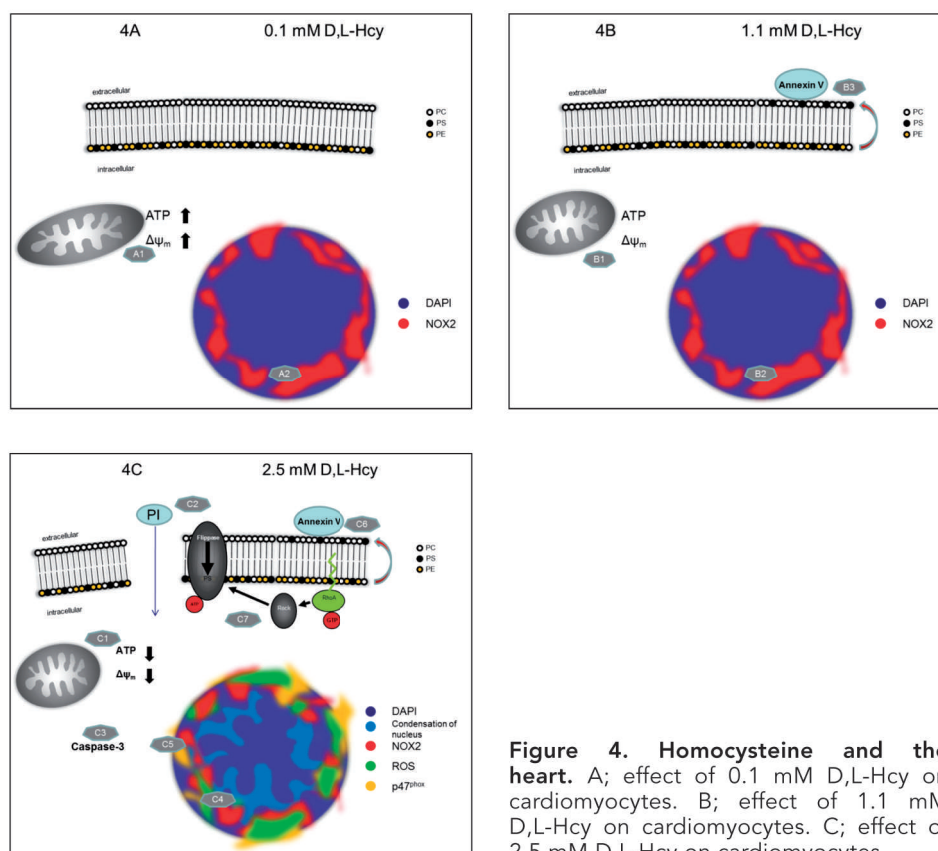


Figure 4. Homocysteine and the heart. A; effect of 0.1 mM D,L-Hcy on cardiomyocytes. B; effect of 1.1 mM D,L-Hcy on cardiomyocytes. C; effect of 2.5 mM D,L-Hcy on cardiomyocytes.

of Hcy was increased up to 1.1 mM D,L-Hcy, a so-called thread-to grain transition within the mitochondria was seen (**B1**), representing an early step in the process of apoptosis⁷⁹. Also then NOX2 translocation to the nucleus (**B2**) was found, although again, without ROS production, nor apoptosis induction. Remarkably, flip-flop induction of the plasma membrane was found (**B3**).

Using 2.5 mM Hcy we again observed a thread-to-grain transition within mitochondria (**C1**), coinciding with a decrease of $\Delta\Psi_m$ and ATP. This supports the concept that Hcy negatively affects the mitochondria in cardiomyocytes⁸⁰ resulting in contractility dysfunction⁸¹⁻⁸³. Even more, we found that 2.5 mM D,L-Hcy induced cell death of cardiomyocytes, namely necrosis (propidium iodide intruding the cell indicative for a permeable plasma membrane (**C2**)) and apoptosis (increased caspase-3 activity (**C3**)). This coincided with (peri-) nuclear NOX2/p47^{phox} expression, and local ROS production (**C4+5**). Similar to 1.1 mM D,L-Hcy, 2.5 mM D,L-Hcy did also induce flip-flop of the plasma membrane (**C6**). This plasma membrane flip-flop does represent a pro-inflammatory status⁴⁵⁻⁴⁷ in cardiomyocytes also. It namely is known that the acute phase protein type IIA secretory phospholipase A₂ (sPLA₂-IIA), which is increased in the blood during heart failure, binds to the flip-flopped plasma membrane. This in turn facilitates binding of the acute phase protein C reactive protein (CRP) to the plasma membrane, that subsequently binds and activates complement, resulting in cell death^{46, 47}. As the plasma membrane flip-flop induction by Hcy represents a new paradigm in Hcy induced cardiotoxicity, we subsequently studied the mechanism(s) of this flip-flop in more detail. In general it is known that PS exposure to the outer leaflet of the plasma membrane is regulated by several factors, including flippase. Flippase is an ATP-dependent transmembrane protein that has been found to regulate transbilayer phospholipid asymmetry also in cardiomyocytes, by translocating PS from the outer to the inner membrane layer⁸⁴. Moreover from studies in megakaryocytes it is known that the low molecular weight GTP-binding protein RhoA also participates in PS exposure⁸⁵. When we examined this in more detail in cardiomyocytes we found in non-Hcy exposed control cells that inhibition of flippase and RhoA, including its downstream effector Rho-associated kinase, resulted in PS exposure⁸⁶. Interestingly, incubation of H9c2 cells during 24 hours with 2.5 mM D,L-Hcy also caused PS exposure via inactivation of flippase and Rho-associated kinase (**C7**).

These results thus indicate that Hcy induced activation of the NOX complex, facilitates apoptosis induction and plasma membrane flip-flop in cardiomyocytes, that is more or less comparable with endothelial cell. However, in contrast to endothelial cells, where already toxic effects were found related to increased levels of SAH alone, this was not the case in cardiomyocytes. Although SAH induced an increase in nuclear NOX2 expression in cardiomyocytes, no local ROS production was found. In line with this, SAH also failed to induce (peri-) nuclear p47^{phox} expression. This expression is necessary in NOX2 mediated ROS production and subsequent apoptosis induction. It therefore suggests partially different pathways in the induction of endothelial dysfunction and cell damage of cardiomyocytes, at least related to SAH.

FUTURE PROSPECTS AND POSSIBLE THERAPIES

Elevated levels of Hcy can be related to a deficiency in co-factors in the methionine metabolism such as vitamin B6, B12 and folate, however the more severe cases are due to genetic mutations in several of the enzymes of the pathway⁸⁷⁻⁹⁰. Since most cases of HHC are due to vitamin shortages the general idea was that supplementing these patients with vitamin B6, B12 and/or folic acid would reduce the risk for cardiovascular events. Over the last four decades large patient trials have been performed to determine whether lowering the Hcy levels in these patients with vitamin supplementation was effective⁹¹⁻⁹⁵. Although Hcy levels were lowered successfully, the risk for cardiovascular events did not reduce significantly in these patients who were already suffering from cardiovascular problems. Therefore there is still some doubt about Hcy being a risk factor in cardiovascular disease. However if we would assume that Hcy caused damage in the early stages of development of cardiovascular disease this would be a logical outcome. Simply taking away the cause after the damage is induced may not be enough to reverse the process. This however still warrants further examination since the duration these patients were treated and studied might be too short to already see a positive effect, similar to glucose lowering trials. In these studies the protective effect to prevent cardiovascular disease were only found after 10 years or more^{96, 97}.

The findings described in this thesis might also offer other therapeutic targets than Hcy itself. We namely have found that both Hcy and SAH resulted in apoptosis in endothelial cells through the expression and translocation of NOX2, p47^{phox} and NOX4 to the (peri)nuclear region, all coinciding with the generation of ROS. In cardiomyocytes only Hcy actually resulted in apoptosis induction whereas SAH failed to induce apoptosis or membrane flip-flop. Therefore a possible target for the development of a therapy could be to inhibit NOX to reduce apoptosis of both cardiomyocytes and endothelial cells, thereby preventing endothelial cell dysfunction and heart failure in HHC patients. Next to this, Hcy and SAH also induced a pro-inflammatory state in endothelial cells through the induction of plasma membrane flip-flop. In cardiomyocytes again, this was only induced by Hcy and not SAH. It is known that Hcy also upregulated pro-inflammatory cytokines such as IL-6 and IL-8, and as such can promote leukocyte recruitment to the endothelium. Moreover, Hcy also increased inflammatory markers in plasma, such as MCP-1 and VCAM-1^{49, 50, 52, 98-103}. Therefore the induction of membrane flip-flop is additionally dangerous, and it would be advisable to measure Hcy levels in patients already having other risk factors for the development of cardiovascular disease, since increased HHC could potentially aggravate the outcome. The other way around, inhibition of inflammatory mediators such as sPLA2-IIA, CRP and complement, theoretically might be beneficial for these patients to reduce inflammatory damage to these cells.

When comparing the role of Hcy or increased SAH in endothelial cells and cardiomyocytes we did find that in cardiomyocytes SAH did not induce p47^{phox} expression, failed to produce ROS, while apoptosis was prohibited. NOX2

translocation by SAH seems to play a role as well, but Hcy itself was the primary jeopardizing factor in cardiomyocytes since it is necessary to complete the process. In endothelial cells increased levels of SAH, without increased Hcy, did induce apoptosis. However, the effect of Hcy alone in endothelial cells and cardiomyocytes was not tested, and we therefore cannot exclude the possible other contributing effects of Hcy on endothelial dysfunction, maybe in a more systemic role. Taken together, this could mean that reduction therapy on SAH could be beneficial to prevent endothelial dysfunction, whereas the prevention of heart failure in HHC patients should be more focussed on lowering Hcy levels.

CONCLUSION

We have found that Hcy induced apoptosis and a pro-inflammatory status not only in endothelial cells but also in cardiomyocytes, coinciding with nuclear NOX-mediated ROS production. As such we provided for the first time evidence that Hcy does have a direct cytotoxic effect on cardiomyocytes. Moreover, we have shown SAH is cytotoxic in endothelial cells, whereas in cardiomyocytes, Hcy is the main culprit, indicative for different cytotoxic pathways in endothelial cells and cardiomyocytes.

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